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DNA FINGERPRINTING OF OIL PALM – CHOICE OF TISSUES

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ABSTRACT

DNA profiling, now commonly used in forensic investigations, can also be used for genetic identification of plants. It is usually stated that for such purpose any tissue from an organism can be used since the DNA is the same throughout. This generalization was tested on the different tissues from oil palm seedlings using 17 PCR-based simple sequence repeat primers. The tissues were those routinely available to a DNA marker laboratory for early screening in a breeding programme. Contrary to the generalization, the different tissues did not always give identical DNA profiles but depended on the primers used. This could be due to dissimilar DNA methylation of the different tissues resulting in quantitative polymorphism detected by some of the primers. While such primers may be useful for tracking changes, such as during development in tissue culture, only the more robust markers would be suitable for routine marker assisted selection in breeding.

Keywords: DNA fingerprinting, oil palm, quantitative polymorphism, profiling.

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INTRODUCTION

DNA profiling is now an indispensable routine in forensic investigation. It is also popular in paternity testing to confirm the parents of a child, especially the father when paternity is uncertain or disputed. The genetic make-up of each organism is unique stemming from its distinctive DNA, hence the term *DNA fingerprinting*. Furthermore, since an organism has the same DNA throughout, it should be possible to use any tissue from the organism for its DNA profile. For example, the DNA of any tissue from a crime scene can be *matched* to ascertain its source. As the genotype is scored directly using DNA markers rather than through expressed genes, there is no restriction to only relatively abundantly expressed structural genes. DNA is stable and resistant to chemicals such as detergents, acids, bases and salt. It is also resistant to environmental degradation and reliable information can even be obtained from very ancient DNA. A classical example is the historical criminal investigation on the last Russian Tsar, where the samples were more than 70 years old (Gill *et al*., 1994). Due to the above-mentioned advantages, DNA profiling is a remarkably powerful and yet versatile tool for personal identification besides applications in population and ecological genetics (Jack *et al*., 1995; Powell *et al*., 1995; UC Davis, 1997; Clegg, 1999; Gurta *et al*., 1999; Benecke and Wells, 2001; Karaca *et al*., 2002; Pal *et al*., 2002; Wiltshire, 2001).

In plants, breeders have applied DNA profiling for fingerprinting genotypes, lines, varieties and cultivars in determining the purity of seed lots, resolving uncertainties in parentage as well as for legal protection of improved varieties through definitive varietal identification (UC Davis, 1997; Kumar, 1999). In a case awaiting trial in the US, prosecutors plan to use cannabis DNA profiles to show that apparently separate cannabis growing operations were actually linked. A database of DNA profiles of different marijuana plants is used to trace the source of any sample and thus, link the user, the distributors and the growers (New Scientist, 2003). DNA-based fingerprinting provides the best currently available technology to establish differences for patent protection and Plant Variety Protection Act Certification (UC Davis, 1997).

In oil palm, genetic fingerprinting systems have been developed for clone identification, specifically to confirm genotypic fidelity between tissue-cultured

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clones and their ortets or palms from which they were derived (Jack *et al*., 1993; 1998). Genetic fingerprinting can also be used in quality control to ensure that clones are not mis-assigned as can happen in culture mix-up when laboratories scale up their work. Since 1996, the Malaysian Palm Oil Board (MPOB) has offered a service to DNA fingerprint oil palm clones using molecular probes (Cheah *et al*., 2000). DNA profiling or fingerprinting can also be used in breeding programmes to assess progenies for spurious pollination (Jack *et al*., 1993; Corley, 2005). In cases where a recording error has occurred, it is often possible to detect the correct parent by matching against the DNA database (Weigel, 2002).

The ability to DNA profile a plant at any developmental stage, including as seeds (Hee *et al*., 1998) or pollen (James *et al*., 2001) makes molecular markers a fast and efficient tool to evaluate cultivar authenticity and purity (Ovesna *et al*., 2002). Rogue plants from errors in pollination and mix-ups during seed handling can be eliminated early during varietal development, hence reducing nursery culling and field selection (Jack *et al*., 1995). This would be particularly valuable in perennial crops like oil palm where field trialling, laboratory testing and data management are costly.

Furthermore, where genetically similar plants are grown in very different environments, say specific DxP crosses or clones on different trial sites, stable fingerprints will allow for reliable tracking of the beneficial traits. In conventional breeding, this is limited by the variable influences of weather and field conditions. Such environmental effects contribute to errors in scoring for the desired gene (Tanskley, 1983). Due to the wide spacing between palms and the long duration, a large oil palm trial will inevitably span a range of environments, making it unlikely that the progenies or clones will rank consistently on phenotypic measures. DNA profiling can unravel the genotypic and environmental components and thus hasten selection progress.

This paper discusses the use of different tissues from small polybag nursery seedlings for DNA profiling using PCR-based simple sequence repeats (SSR) primers or markers.

MATERIALS AND METHODS

Materials

From a batch of 100 three-month-old oil palm seedlings, the following tissues were obtained - roots (R), green leaves (L), haustoria (E), kernels (K) and shell (S). The 17 primers used were PCR-based, simple sequence repeats (SSR) from microsatellite and retrotransposon analysis.

Methods

DNA was extracted from the different tissues mentioned above using the modified DNA extraction method of Dellaporta *et al.* (1983). The tissues were collected separately from each of the 100 seedlings, bulked according to tissue type and manually ground into powder in liquid nitrogen. About 2 g of each ground tissue were then used for DNA extraction. For each tissue, DNA extraction was done twice (two replicates), for example, R1, R2. In the case of the shell, as the tissue is hard, it was first broken into smaller pieces using a basic microfine grinder (Model MF10, IKA) and only then ground into fine powder in liquid nitrogen. As the extracted shell DNA was contaminated with lignin, the two replicates of shell DNA were bulked into one sample (S1) for purification by ethanol precipitation. DNA profiling was carried out by PCR using the 17 primers. The PCR products were analysed by gel electrophoresis using 2.5% metaphor agarose gel stained with ethidium bromide, running at 80V and viewed under ultraviolet light. Primers that gave multiple bands were further analysed using 8% polyacrylamide gel stained with ethidium bromide. The presence or absence of bands on the gels were scored '1' and '0' respectively (*Table 1*).

RESULTS AND DISCUSSION

Of the various tissues tested, the shell was the most problematical in the DNA extraction and profiling. The stone cells that dominate the tissue are difficult to crush by conventional methods. Long milling was necessary but the heat generated may denature the DNA. Only the reaction with two primers, A36 and A8, gave bands for the DNA, the former showing a single band and the latter two bands.

Compared to the shell, only small amounts of DNA were extracted from the kernel. During DNA isolation, white sediment was observed when the kernel DNA was dissolved in the final solution, Tris/EDTA (TE) buffer. When the PCR products were mixed with the blue loading dye, the colour was of lighter blue than those of the DNA mixtures of other tissues. When the mixture was loaded into the gels, most of the solution floated in the running buffer instead of sinking to the bottom of the wells of the gels as is usually the case. This was probably due to the DNA mixture containing oil released during DNA isolation. Both the shell and kernel tissues gave poor DNA profiles as bands were hardly observed with almost all primers (*Table 1*).

Although the haustoria were also superficially oily, there were less white sediment and floating of the loading mixture as the spongy haustorial tissue is mainly filled with the breakdown products from

TABLE 1. BANDING PATTERNS OBTAINED FROM DIFFERENT TISSUES TESTED WITH DIFFERENT PRIMER

Sample	A4	A8 (P)	A12 (P)	A13 (P)	A19 (P)	A21	A28	A31	A33	A36 (P)	A37	B1 (P)	B4 (P)	C3	C6	C11(P)	C12(P)
Root 1	1	11110	01111110	111	1111	1	011	111111111	1	11101100100	11	1111111	1111111	11	111	1111	10111
Root 2	1	11110	01111110	111	1111	1	011	111111111	1	11101100100	11	1111111	1111111	11	111	1111	10111
Leaf 1	1	00110	11111101	101	1111	1	111	001111111	1	11111111111	11	1111111	1111111	11	111	0111	11111
Leaf 2	1	00110	11111101	101	1111	1	111	001111111	1	11111111111	11	1111111	1111111	11	111	0111	11111
Haustoria1	1	11111	01101100	001	1010	1	010	000000111	1	10000100100	11	0010110	0001001	11	010	1011	00011
Haustoria2	1	11111	01101100	001	1010	1	010	000000111	1	10000100100	11	0010110	0001001	11	010	1011	00011
Kernel 1	0	00001	-	0	-	0	0	0	0	0	00	0	0	0	0	0	
Kernel 2	0	00001	-	0	-	0	0	0	0	0	00	0	0	0	0	-	
Shell 1	0	00001	-	0	-	0	0	0	0	10000000000	00	0	0	0	0	0	
Shell 2	0	00001	-	0	-	-	0	0	0	-	-	0	0	-	0	-	

Notes: *The '0's and '1's correspond to the absence or presence, respectively, of bands when the PCR products of sample DNA and primer are separated on gels.
2- P = separation on polyacrylamide gel.

the oil-rich kernel. More DNA was isolated from the haustoria than from the kernels as indicated by the more intense DNA bands for haustoria on gel electrophoresis (*Figure 1*). The PCR for haustoria also produced better profiles than that for the kernels (*Table 1*).

From the bands produced in *Table 1*, except for the shell and kernel tissues which could hardly be profiled, primers A4, A21, A33, A37 and C3 gave similar profiles for all the other tissues. The first three produced single band while primers A13 and C3 gave two bands profiles (*Figure 2*). However, the other 12 primers showed different profiles with different tissues. There were no systematic differences. More fragments were generated when the root and shoot DNA were primed compared to those from the shell and kernel DNA. The roots and shoots were bulked from the open-pollinated individuals of a single F1 mother palm (P1), similar to a *tenera* x *tenera* F2 while shells are maternal tissues (P1) and kernels are triploid and more representative of P1. As shown in *Table 1*, only primer A8 gave two bands for both the shell and kernel tissues. These results were not surprising as the mother palm, P1, did not show many bands when its leaf DNA was profiled with the same markers (results not shown).

The differences in banding patterns between the tissues, between roots and shoots for example, were due to some bands being absent (*i.e. missing* bands) in certain tissues. Arnholdt-Schmitt *et al.* (2001) reported that quantitative polymorphism or differences in the intensity of the amplified fragments could mask the less intense bands, giving a false *absence* which may be erroneously interpreted as qualitative polymorphism (Arnholdt-Schmitt *et al*., 2001; Schaefer *et al*., 2000; Arnholdt-Schmitt, 2003). It was noted that the differentiation stage and physiological state of cells and tissues may result in quantitative polymorphism (Arnholdt-Schmitt *et al*., 2001). In plants, during cell or tissue development and differentiation, DNA methylation plays an important role in regulation of gene expression (Li, 2002; Finnegan *et al*., 1998). Close correlations between the repeated DNA sequences and their methylation status (Arnholdt-Schmitt *et al*., 1991) as well as the preferential accumulation of methylated cytosine (m5c) in repeated DNA sequences have been widely reported (Deumling, 1981; Sturm and Taylor, 1981; Ehrlich *et al*., 1982; Pages and Roizes, 1982; Arnholdt-Schmitt *et al*., 1991; Ehrlich *et al*., 1981; Martienssen *et al*., 2001; Finnegan *et al*., 1998). Since the primers used in this study were SSR, *i.e*. repeated sequences, and such sequences are relatively rich in methylated sequences, especially methylated cytosine, it is possible that the quantitative polymorphism observed arose from methylation of the sequences during tissue differentiation and development in the rapidly growing seedlings. Methylated sequences are reported to affect DNA or RNA synthesis by influencing interactions with sequence specific proteins (Ehrlich *et al*., 1981). They influence DNA template activity by affecting denaturation of the DNA as methylation of a specific C or A residue in the DNA recognition site prevents hydrolysis at the methylated sequence (Arber, 1979). Furthermore, the *tenera* type of oil palm (as used in this study) has been reported to be more methylated than other fruit types (Shah *et al*., 1992).

This study illustrates the importance of using the same tissue from each sample in comparative DNA profiling work. Leaves would normally be the tissue of choice as they are easily harvested without much detrimental effect to the seedlings. Roots would be less frequently used as they are more difficult to harvest and clean. The exceptions may be plants in tissue culture and very tall palms which leaves may be difficult to obtain making it more convenient to induce fresh roots at their stem bases. The kernel is the earliest progeny tissue available for DNA profiling a plant but its triploid nature must be kept in mind when interpreting the results. Unless the seeds or kernels are stored well, contamination from microbial DNA may be a problem. The above two problems may be obviated by taking haustorial tissue from germinating seeds. Once established in soil, the seeds can be broken off to extract the remnants of the haustoria for DNA work while allowing the seedling to continue its growth. Shells, especially of *dura* palms, are the toughest and, hence, longest lasting tissues. Indeed they may be the most conveniently collectable tissue on the ground; for example during plant expeditions or when the fruits are not available or the palms are too tall. The *seeds* on the ground are mostly empty shells with their kernels rotted away. Of course, the caveat is that the shells may not be from the nearest palms where they were found.

With the above in mind, although leaf tissue is the most widely used for DNA profiling, other tissues such as roots, kernels, shells (maternal tissues) and haustoria may have to be used in particular circumstances such as for quality control in seed production and tissue culture.

The 17 PCR-based SSR primers used were designed from microsatellite and retrotransposons to target repetitive DNA sequences which are abundant, especially in the non-coding regions of the plant genome (Jarne *et al*., 1996; Heslop-Harrison, 2000; Ovesna *et al*., 2002; Bilotte *et al*., 2001; Melinek *et al*., 2002). These repeats are naturally highly variable, making them good choices as markers for profiling work (Jarne *et al*., 1996; Melinek *et al*., 2002; Schlotterer, 2004). Good DNA profiles are obtained when a substantial number of fragments of various sizes assort into polymorphic DNA banding patterns

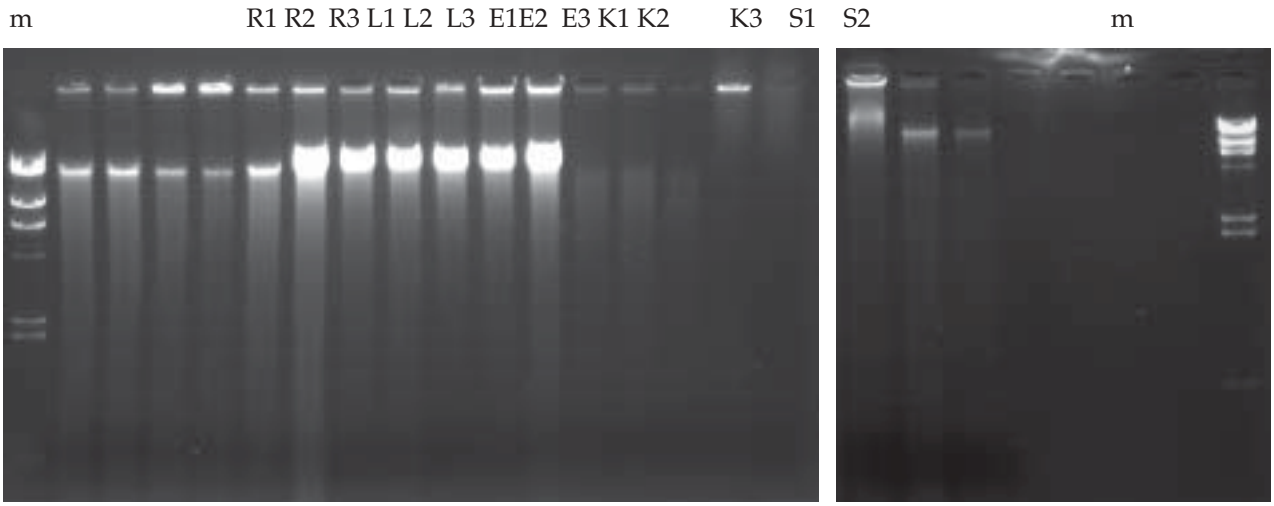

Figure 1. Genomic DNA of the different tissues. The genomic DNA was extracted from each tissue type and electrophorized on 0.8% agarose gel at 50V, before staining with ethidium bromide for viewing on an UV light box. Each tissue was replicated in two or three batches. R1-R3: replicates of root tissue; L1-L3: replicates of leaf tissue; E1-E3: replicates of haustorium; K1-K3: replicates of kernel; S1-S2: replicates of shell. The standard DNA marker (m) used was lambda HindIII DNA ladder.

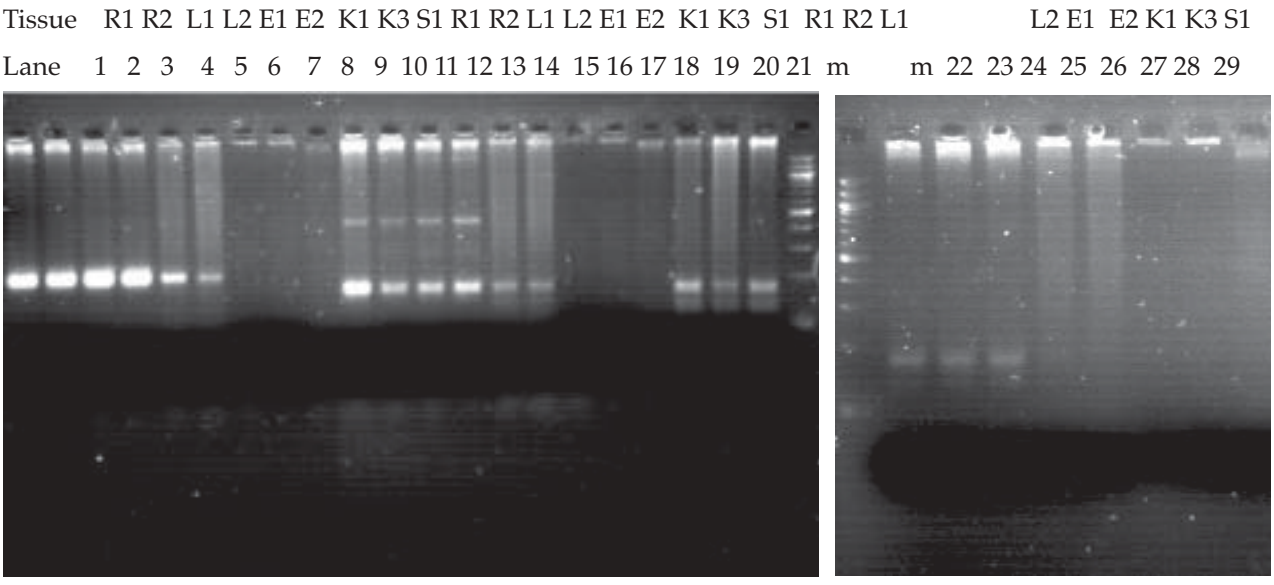

Figure 2. DNA profiles of the different tissues with different primers on 2.5% metaphor agarose gel stained with ethidium bromide. The primers used were A21 (lanes 1- 9), A13 (lanes 10- 18), and C3 (lanes 19-27). The standard DNA marker (m) was a 100 bp DNA ladder.

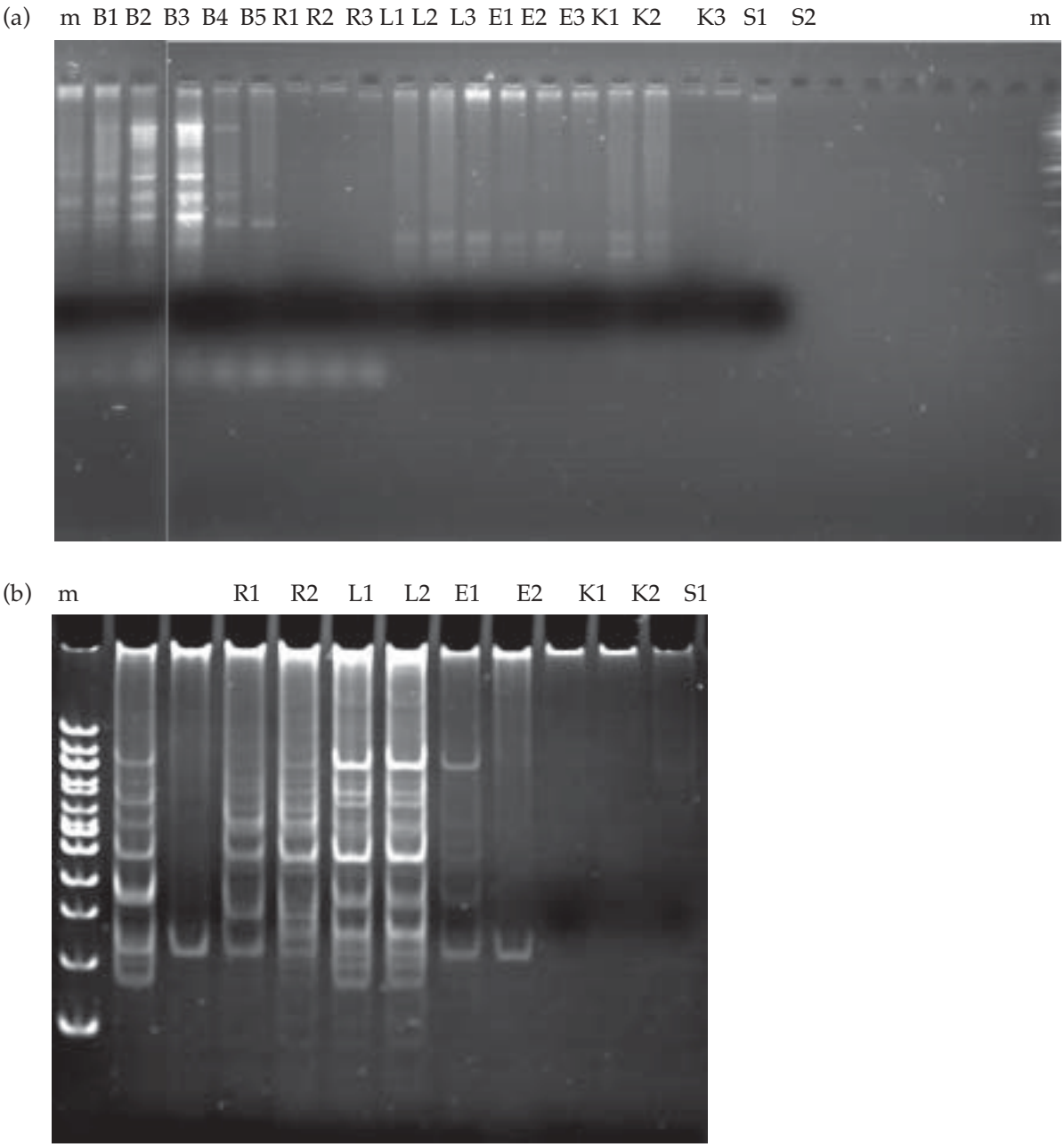

Figure 3. DNA profiles of the different types of oil palm tissues [root (R), leaf (L), haustorium (E), kernel (K) and shell (S)] with primer A36 on (a) 2.5% metaphor agarose gel and (b) 8% polyacrylamide gel respectively. The gels were stained with ethidium bromide before viewing under UV light. The standard DNA marker (m) used was a 100 bp DNA ladder.

(*DNA fingerprints*) when separated by gel electrophoresis. However because of the abundance of repeated sequences (50%-90%) in the plant genome (Heslop-Harrison, 2000), microsatellite primers generate many fragments that do not separate well on normal agarose gel. Furthermore, many of the fragments may be common in all the tissues such as the housekeeping DNA in well conserved regions of the genome. Hence, for high throughput screening, as is required, for example in marker assisted selection, the SSR fragments from retrotransposon and microsatellite analysis are converted into PCR-based primers (Mohan *et al*., 1997; Joshi *et al*., 1999; Kumar, 1999; Schlotterer, 2004; Pal *et al*., 2002). These PCR-based primers, designed from the flanking regions of the SSR loci yield highly polymorphic, yet unique fragment(s) during PCR (Mohan *et al*., 1997; Joshi *et al*., 1999, Jobling and Gill, 2004; Holton, 2001). However, PCR-based primers that generate only a few bands, such as A21 and A4 in this study which generated only single bands must be tagging specific regions in the genome. Such primers are very useful if linked to traits of interest that segregate clearly in fixed ratios. The presence/

absence of such bands is easily scored and the problem of mis-scoring or errors during visualisation of the gel results is minimal. Multiple bands, especially if less than 10% of bases apart are more difficult to score and even more so if done manually. Where good separation is not obtained with metaphor agarose, acrylamide gel can be used. The latter can resolve nucleotide differences as small as one base pair (bp) whereas a 4% metaphor gel can only detect size differences (in bp) of about 2% (Senior *et al*., 1998). The higher resolution of acrylamide gels allows detection of a larger number of alleles per locus. This may be seen in *Figure 3* where the bands are better seen on 8% polyacrylamide gel than on 2.5% metaphor agarose gel. For high throughput, SSR can be further improved by running the PCR reactions using automated capillary electrophoresis systems with fluorescence detection. However, one must be aware that the fragments detected as peaks could be stuttering bands generated from the sensitivity of PCR (Warburton *et al*., 2002; Holton, 2001). The errors from the high sensitivity and profile mis-interpretation can be minimized by screening the primers on as many populations as possible.

CONCLUSION

DNA profiling is a powerful technique that can benefit oil palm breeding. However, careful thought and preliminary work are necessary for good primer design and testing. Circumstances, not always choice, may dictate the type of tissue to be used for analysis. Although the DNA is the same throughout an organism, the results of this study demonstrate the presence of quantitative polymorphism which can be elicited by some markers when using different tissues of the same organism. In other words, the different tissues from the same plant may not necessarily give the same DNA profile. Primers which result in consistent patterns, irrespective of the tissues used, would be the most suitable for augmenting breeding work. Primers that show different patterns in different tissues should be tested for their value in tracking changes during growth and differentiation. Such primers may have a role in monitoring changes in the plant genome and could be developed, for example, for quality control in tissue culture. When working with seeds, the shell, haustorium and kernel can be used for DNA profiling using normal preparation methods.

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